The Immunopathologic Effects of *Mycoplasma pneumoniae* and Community-acquired Respiratory Distress Syndrome Toxin A Primate Model

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Abstract

Mycoplasma pneumoniae infection has been linked to poor asthma outcomes. *M. pneumoniae* produces an ADP-ribosylating and vacuolating toxin called community-acquired respiratory distress syndrome (CARDS) toxin that has a major role in inflammation and airway dysfunction. The objective was to evaluate the immunopathological effects in primates exposed to *M. pneumoniae* or CARDS toxin. A total of 13 baboons were exposed to *M. pneumoniae* or CARDS toxin. At Days 7 and 14, BAL fluid was collected and analyzed for cell count, percent of each type of cell, CARDS toxin by PCR, CARDS toxin by antigen capture, eosinophilic cationic protein, and cytokine profiles. Serum IgM, IgG, and IgE responses to CARDS toxin were measured. All animals had a necropsy for analysis of the histopathological changes on lungs. No animal developed signs of infection. The serological responses to CARDS toxin were variable. At Day 14, four of seven animals exposed to *M. pneumoniae* and all four animals exposed to CARDS toxin

developed histological “asthma-like” changes. T cell intracellular cytokine analysis revealed an increasing ratio of IL-4/IFN- γ over time. Both *M. pneumoniae* and CARDS toxin exposure resulted in similar histopathological pulmonary changes, suggesting that CARDS toxin plays a major role in the inflammatory response.

Keywords: asthma; mycoplasma; community-acquired respiratory distress syndrome toxin; primate model

Clinical Relevance

This study provides further understanding of the role of community-acquired respiratory distress syndrome toxin and *Mycoplasma pneumoniae* in the inflammatory response of this infection in the lung.

Asthma is a complex inflammatory disease of the lungs that has reached epidemic proportions in the developed world. It is estimated to affect 300 million worldwide and 10% of the population in the United States and Europe (1, 2). Both viral and

bacterial infections are felt to contribute to the etiology and exacerbation rate of asthma. Early reports demonstrated an association between *Mycoplasma pneumoniae* and asthma symptoms (3, 4). More recently, atypical bacterial infections

or colonization with *M. pneumoniae* have been increasingly associated with the development of asthma and the worsening of asthma control (5–7). Both human and animal studies suggest that *M. pneumoniae* may play an important role in both the

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initiation and progression of chronic asthma (8–12). Even in individuals without atopy, *M. pneumoniae* infection has been linked to the development of asthma in both early and late stages (13). Furthermore, therapies targeting *M. pneumoniae* have shown potential beneficial effects in asthma control (14).

Until recently, no virulence factors of *M. pneumoniae* have been directly linked to asthma pathogenesis. We identified an ADP-ribosylating and vacuolating toxin produced by *M. pneumoniae*, community-acquired respiratory distress (CARDS) toxin, which is strongly linked with *M. pneumoniae*-associated disease in humans and directly linked to asthma-like inflammatory patterns in animal models (15–17). In children, *M. pneumoniae* colonization is seasonal, and CARDS toxin is readily detected in the respiratory secretions of children in the winter months. In fact, approximately 50% of hospitalized children with acute exacerbation of asthma had detectable CARDS toxin in their respiratory secretions (11). This is in agreement with what we reported in adults with refractory asthma, where 52% of the subjects with refractory asthma had detectable CARDS toxin in the respiratory secretions (18). Importantly, within this patient group, we observed a subset of patients who were persistently positive for CARDS toxin for up to 600 days.

Despite these observations suggesting a link between asthma and CARDS toxin, there is still limited understanding on the inflammatory mechanisms of CARDS toxin and its effect on the development of asthma and its control. The objective of this study was to further characterize the immunological and histopathological response to *M. pneumoniae* infection and exposure to CARDS toxin using a primate model.

Methods

A total of 13 adult baboons (*Papio* sp.) was used for the experiment. Animals were individually housed and had free access to food, water, and toys. After anesthesia, all baboons underwent bronchoscopy using an Olympus P20-D bronchoscope (Olympus Medical Systems Corp.). The animals' vital signs (heart rate, blood pressure,

temperature, and oxygen saturation) were monitored closely before and after the experiment. The animals were weighed daily, measured for nutritional and fluid intake, and examined twice daily for normal interactions. The baboons were treated in accordance with guidelines established by the Weatherall report (19). All experiments were performed in accordance with the Institutional Biosafety Committee and Institutional Animal Use and Care Committee protocols of the University of Texas Health Science Center at San Antonio (San Antonio, TX).

All animals had baseline serum drawn, and underwent bronchial lavage with instillation of 20 ml of saline solution, with a return of 10 ml of BAL fluid. After the acquisition of the baseline BAL fluid from the left lower lobe, *M. pneumoniae* (10^8 color-changing units/ml; $n = 9$) or CARDS toxin (5 mg/10 ml; $n = 4$) was instilled into the bronchus intermedius. This dose was based on the concentration of toxin per milliliter of secretions detected in patients with severe asthma and the estimated volume of distribution. *M. pneumoniae* strain S1, grown on SP4 liquid media, was used. The process of production and purification of recombinant CARDS toxin has been described previously (15).

Serum and BAL fluid were collected again at Days 7 and 14 after inoculation. All samples of BAL fluid were analyzed for cell count, percent of each type of cell, PCR for CARDS toxin, CARDS toxin by antigen capture, and eosinophilic cationic protein, as previously described (16–18). Animals with *M. pneumoniae* exposure had bronchoscopic microsampling of the epithelial lining fluid (ELF) on Day 7 using the Olympus microsampling probe (model BC-402; Olympus Optical Co., Ltd.) at the bronchus intermedius. At Day 7, four animals underwent necropsy (two of the *M. pneumoniae* group and two of the CARDS toxin group), with the remaining animals undergoing necropsy on Day 14.

Pathology Methods

After intrabronchial fixation with 10% neutral-buffered formalin, both right and left lower lung lobes were cut transversely into two levels, the surfaces of which yielded four tissue specimens from each lobe for paraffin processing. Sections were cut at 4 μ m and stained with hematoxylin and eosin. All slides were scanned with the Aperio Scanscope XT (Aperio) to create

digital images. The hematoxylin and eosin digital images were reviewed in a blinded fashion, and lesions of peribronchiolar and bronchial infiltrates, bronchiolar and bronchial luminal exudates, perivascular infiltrate, and parenchymal pneumonia were evaluated. This method assigns values from 0 to 26 (the greater the score, the greater the inflammatory changes in the lung). Inflammatory cell infiltrates of lymphocytes and polymorphonuclear leukocytes were graded at few (grade 1), numerous (grade 2), or abundant (grade 3) in peribronchial/peribronchiolar, perivascular, and intra-alveolar pneumonitic exudate sites.

A representative slide from both the right and left lung of all animals was immunostained using a monoclonal mouse antibody against eosinophil peroxidase (EPX) (provided by J. Lee, Mayo Clinic, Scottsdale, AZ) at a dilution of 1:100 to identify eosinophils. Using the Aperio image analysis system, image analysis algorithms were used to obtain unbiased estimates of EPX-immunostained cells. Anti-rabbit recombinant CARDS toxin column affinity-purified antibody at a 1:1,200 dilution was incubated with representative lung sections, which were then stained with diaminobenzidine chromagen. The findings were correlated with light microscopic histopathological lesions of pneumonia and airway and vascular cellular infiltrates.

Serology

Serum IgM and IgG responses to CARDS toxin were determined by direct ELISA. ELISA plates were coated with recombinant CARDS toxin, 85 ng/well for IgM and 340 ng/well for IgG assessment, and then incubated with baboon serum diluted 1:200. After extensive washing, relative IgM and IgG concentrations were determined using peroxidase-conjugated goat anti-baboon IgM and IgG antibodies and detected using colorimetric substrate. The optical density (OD) at 630 nm was measured on a plate reader and the mean of duplicate measurements reported. An OD at 630 nm above 0.2 was considered above background.

An IgE capture was developed as an ELISA for measuring CARDS toxin-specific IgE antibody in serum. Microplate wells (Thermo Immulon HBX4; Thermo Fisher Scientific) were first coated with a commercial anti-human IgE monoclonal antibody (Kirkegaard and Perry

Laboratory), and then incubated with serial dilutions of test sera with 1% BSA as diluent for the capture of only IgE-class antibodies. CARDS toxin plus anti-CARDS toxin-specific rabbit polyclonal antibodies and alkaline phosphatase-conjugated secondary antibody were then added. Finally, p-NPP (Sigma-Aldrich) was used as the enzyme substrate. The OD of the reaction product was measured at 405 nm on a microplate reader.

Flow Cytometry

Flow cytometry was performed in the Core Laboratory of the University of Texas Health Science Center at San Antonio. Peripheral blood mononuclear cells were obtained using Ficoll-Hypaque density gradient centrifugation. Cells were then incubated and then washed with 0.5% Pharmingen (BD Biosciences Pharmingen) stain buffer, and 0.1% NaN₃ in PBS. Cells were then blocked with 5% mouse serum and 10% human Fc fragments, incubated with subsequent washing, permeabilized with 50% diethylene glycol and 15% formaldehyde (Permeabilizing Solution 2; BD Biosciences), blocked with 10% normal mouse serum and 20% human Fc fragments, and stained with monoclonal antibodies (CD3 v500 [UCHTL1], CD4-APC-Cy7 [RPA-T4], CD8-PE-Cy7 [RPA-T8], IL4-PE [8D4-8], IL17-APC [eBio64Dec17], IFN-FITC [B27]) against intracellular antigens. Dead cells were discriminated using Violet Dead stain (Thermo Fisher Scientific).

Results

Clinical Response to CARDS Toxin and *M. pneumoniae*

No animal developed clinical signs of infection, and all animals maintained normal activity throughout the experiment (see Table E1 in the data supplement). The endoscopic exam appeared normal and without secretions after exposure to either CARDS toxin or *M. pneumoniae* on Days 7 and 14.

Histopathology and Immunostaining Results

The grades of scanned images and a summary of each animal's histopathology are presented in Table 1. Overall, the animals with the least visually identified lesions were those exposed to *M. pneumoniae* at Day 7 (AD12, FA52) and

Day 14 (AD67). Conversely, at Day 7, the two CARDS toxin-exposed animals had moderate grades of disease involvement and, at Day 14, both CARDS toxin-exposed baboons had severe lung disease grades. Only two of the Day 14 *M. pneumoniae*-exposed animals developed severe lesions (FA03, FK73), whereas other *M. pneumoniae*-treated animals had moderate grades of disease. Figure 1 depicts examples of the mild, moderate, and severe grades of disease in CARDS toxin- or *M. pneumoniae*-exposed right lung lobes and its left lung control.

All animals exposed to CARDS toxin at Days 7 and 14 developed "asthma-like" histopathologic changes characterized by significant lymphocytic and eosinophilic airway disease with more than five eosinophils per 20× field and mucus plugging (Table 1). On Day 14, one animal (AN53) had severe multilayering of lymphocytes and eosinophils around the vessels and airways. The EPX value on this animal was 1.16%. The left lung was histologically normal. After instilling CARDS toxin endobronchially to EV36, the animal was noted to vomit, and was turned on his left side and suctioned. The animal subsequently developed significant disease in both lower lobes (Table 1). EV36 developed greater than five eosinophils per 20× field in both lungs, with a slight increase in polymorphonuclear leukocytes. The EPX value on this animal was 4.37% on the right and 2.23% on the left.

At Days 14, four of seven animals exposed to *M. pneumoniae* (BJ14, FK73, AC86, and FA03) developed "asthma-like" histopathologic changes (Figure 1, Figures E1 and E2). Only one animal (AC86) had eosinophils on baseline BAL (1%), and this value increased to 30% at the time of necropsy. As noted in the summary of lesions in Table 1, this animal had histologic signs of aspiration and was also noted to have 20% polymorphonuclear leukocytes in both the right and left lung BAL at necropsy (data not shown). Another animal (FA03) had asthma-like disease with elevated EPX levels determined by the Aperio system (13.75% of cells were eosinophils in the right lung and 0.70% in the left lung). This animal also demonstrated focal eosinophilic pneumonia and an area of mucus plugging in the left lung. Although this animal had only scattered eosinophils in the walls of distal

airways, abundant eosinophils in the wall of the large bronchus plus the focal sites of eosinophilic pneumonia were likely contributors to the EPX value. Figures E1A, E1B, and E2A depicts the multilayering of lymphocytes in both *M. pneumoniae*- and CARDS toxin-treated infected lungs; of note is the striking eosinophilic presence in CARDS toxin-treated lung at 14 days (Figure E2B) and the EPX stain for eosinophils at 7 days after CARDS toxin treatment (Figure E2C).

The EPX data consistently show higher values in the right lung when compared with those in the left lung (Table 1). By Day 14, five of seven *M. pneumoniae*-exposed animals demonstrated higher EPX values (range, 2- to 20-fold) when compared with those of left lung. Three had eosinophils within the alveoli that were likely contributors to higher EPX values. All CARDS toxin-treated animal lungs had consistently higher right-lobe EPX values (range, 2- to 26-fold) and numerous to abundant eosinophils in the walls of distal airways and vascular structures. Because animals were noted to cough after extubation, the histological findings on the left lung were not unexpected. Immunostaining with CARDS toxin antibody was present in alveolar macrophages in peribronchiolar/alveolar spaces in CARDS toxin-treated animals at Day 14.

Serological Responses to CARDS Toxin

Animals exposed to recombinant CARDS toxin or infected with *M. pneumoniae* had variable serological responses (Figure 2). Three animals (BJ14, FK73, and AD93) showed CARDS toxin-specific IgM at baseline, and four animals (AC86, AD67, FK73, and FA52) had low, but detectable, levels of CARDS toxin-specific IgG. Of note, 75% of the animals treated with recombinant CARDS toxin showed increases in both toxin-specific IgM and IgG over the course of the experiment, whereas only 22% of the *M. pneumoniae*-infected animals showed increased IgM, and 44% had increases in IgG. Overall, animals exposed to recombinant CARDS toxin had better IgM and IgG responses than animals infected with *M. pneumoniae*. One animal (AC86) had increased IgG without a corresponding change in IgM. Four animals (DA84, DL98, AD12, and BJ14) did not increase IgM or

Table 1. Exposure Status, Histopathology, Number of Lung Slides with Disease/Grade, Percent of Eosinophil Peroxidase Cell Staining, Community-acquired Respiratory Distress Syndrome Toxin by Antigen Capture in *Mycoplasma pneumoniae*-infected Animals, and IgE Community-acquired Respiratory Distress Syndrome before and after Exposure

Animal	Exposure	Day of Necropsy	Histopathology	Right Lung	Left Lung	Right Lung EPX (%)	Left Lung EPX (%)	CARDS Antigen Capture	IgE CARDS Before (After)
ET92	CARDS toxin	7	Asthma-like disease	2/4 moderate	0/4	4.52	0.18	12,790	0.047 (0.072)*
DA84									
EV36		14	Asthma-like disease	3/4 moderate	2/4 mild	1.03	0.34	1,991	0.036 (0.1)
AN53			Asthma-like disease, focal eosinophilic pneumonia	4/4 severe	4/4 severe	4.37	2.23	334	0.062 (0.232)
AD12	Mp	7	Minimal disease with focal sites of lymphocyte aggregates and rare eosinophils in airway and vessel walls	4/4 severe	0/4	1.16	0.37	157	0.082 (0.112)
FA52			Minimal disease with focal sites of lymphocyte aggregates and rare eosinophils in airway and vessel walls	1/4 mild	0/4	0.36	0.30	348	0.035 (0.083)*
DL98		14	Multilayered lymphocytes and rare eosinophils in airway and vessel walls	4/4 moderate	2/4 mild	0.37	0.34	1,739 [154]	0.069 (0.125)
BJ14			Asthma-like disease	2/4 moderate	0/4	0.81	0.43	268 [34]	0.047 (0.096)
FK73			Asthma-like disease, focal eosinophilic pneumonia	3/4 severe	0/4	1.24	0.36	1,471 [48]	0.042 (0.116)
AD93			Multilayered lymphocytes and rare eosinophils in walls of vessels and airways	3/4 moderate	0/4	0.43	0.21	670 [73]	0.084 (0.123)
AC86			Asthma-like disease, eosinophilic pneumonia, and focal foreign body giant cell lesion	3/4 moderate	1/4 mild	2.13	0.36	247 [469]	0.032 (0.139)
AD67			Focal sites of lymphocytes and rare eosinophils in walls of vessels and airways	1/4 mild	1/4 mild	0.30	0.24	298 [64]	0.059 (0.125)
FA03			Asthma-like disease mixed cell pneumonia	2/4 severe	0/4	13.75	0.70	1,252 [141]	0.036 (0.078)*

Definition of abbreviations: CARDS = community-acquired respiratory distress syndrome; EPX = eosinophil peroxidase; Mp = *Mycoplasma pneumoniae*.

Asthma-like disease: significant lymphocytic and eosinophilic airway disease with more than five eosinophils per 20× field and mucus plugging. *Mycoplasma* animals have antigen capture (pg/ml) shown at both Day 7 and Day 14 (in brackets). IgE CARDS toxin by antigen capture was measured before exposure and after killing, and considered positive if greater than 0.09 optical density at 450 nm.

*Negative postmortem IgE CARDS.

Figure 1. (A) Mild (<50%) grade of multilayered cellular infiltrates reflected visually in scanned lung section; lesions with fewer cell infiltrates are not evident in the scans. (B) Moderate (50–75%) grade of multilayered cellular infiltrates reflected visually in scanned lung section. (C) Severe (75–100%) grade of multilayered cellular infiltrates reflected visually in scanned lung section. Treated right lower lobe (upper panel) and “untreated control” left lower lobe (lower panel) of the same animal are illustrated in each case. CARDS = community-acquired respiratory distress syndrome; LLL = left lower lobe; MP = *Mycoplasma pneumoniae*; RLL = right lower lobe.

IgG titers above baseline throughout the course of the experiment.

All animals had negative IgE to CARDS at baseline and, with the exception of three animals (ET92, AD12, and FA03), developed IgE to CARDS by the time they were killed. IgE levels did not correlate with CARDS toxin levels or eosinophil counts (Table 1).

Intracellular Cytokine Staining of Peripheral Blood Lymphocytes

Intracellular cytokines were evaluated on Days 0 and 7 for all animals, and on Day 14 for those animals not killed on Day 7. Intracellular IL-4, IL-17, and IFN- γ were reduced in T cells (CD3⁺) on Days 7 and 14 after either CARDS toxin or *M. pneumoniae* inoculation (Figure 3). The mean reduction at Day 7 was 22.4% for IFN- γ , 90.6% for IL-4, and 43.1% for IL-17. However, by Day 14, IL-4 rebounded toward baseline in five of seven animals, whereas IFN- γ continued to decline. The ratio of IL-4:IFN- γ , although initially reduced at Day 7 (mean, 0.145) compared

with Day 0 (mean, 0.225), increased at Day 14 (mean, 0.230). However, two CARDS toxin-treated animals with asthma-like lesions (EV36 and ET92) had very little change in their IL-4:IFN- γ ratio on Day 7 (0.06 and –0.1).

Production of CARDS Toxin in Animals Exposed to *M. pneumoniae*

All *M. pneumoniae*-infected animals had measurable CARDS toxin on Days 7 and 14 by antigen capture (Table 1). With the exception of one animal, all levels of CARDS toxin were lower at Day 14 (mean, 849.1 $\pm$ 629.6 versus 140 $\pm$ 151.9 pg/ml; $P = 0.013$). Five of seven animals had measureable CARDS toxin in the ELF on Day 7. The mean level of CARDS toxin was 353.5 pg/ml (range, 11.7–912.7 pg/ml) in ELF, which is comparable to levels we detected in patients with severe/refractory asthma. The degree of histologic severity was correlated to level of CARDS toxin by antigen capture on Day 7 ($r = 0.676$, $P = 0.046$), but not on Day 14 ($r = 0.306$, $P = 0.504$).

Discussion

The results of this study demonstrate that both *M. pneumoniae* and CARDS toxin exposure are capable of producing asthma-like histopathologic changes in primates and IgE to CARDS toxin. All CARDS toxin-treated animals had asthma-like changes on histologic exam. On Day 7, *M. pneumoniae*-exposed animals had mild lymphocytic reactions around vessels and airways, but only rare eosinophils, but by Day 14, progression of the lymphocytic and eosinophilic responses was more robust and “asthma like” in four of seven animals. In general, the number of eosinophils reflected in the EPX values and documented histopathological findings correlated with the severity and extent of disease in the digital images.

Based on clinical observations, we developed animal models of CARDS toxin-induced inflammation. Our previous studies demonstrated that recombinant CARDS toxin induces peribronchiolar inflammation in both mice and baboons (16).

A **B**

Figure 2. (A) IgM antibody response to CARDS toxin and *Mycoplasma pneumoniae* organisms as determined by ELISA. (B) IgG antibody response to CARDS toxin and *M. pneumoniae* organisms as determined by ELISA. OD = optical density.

Subsequent studies in mice demonstrated that CARDS toxin induces asthma-like inflammation (17). This inflammation consists of a robust eosinophilia, induction of IL-4 and IL-13 expression, mucus metaplasia, and airway hyperreactivity. It was demonstrated that this response is dependent on CD4⁺ T cells, and shows a trend of polarization toward a type-2 inflammation pattern. Altogether, these data suggest that CARDS toxin can induce allergic inflammation in rodents.

In our mouse models, treatment with recombinant CARDS toxin or exposure with *M. pneumoniae* resulted in an early (by Day 7) and robust polarization of the T cell response to a type-2 phenotype (16, 17, 20). In contrast, the response in baboons was delayed, and demonstrated a mixed immunologic response characterized by a decline in IL-4, IL-17, and IFN- γ by Day 7, with a trend for IL-4 to return toward baseline, whereas IL-17 and IFN- γ continued to decline in the majority of

animals. This resulted in an increase in the IL-4:IFN- γ ratio, suggesting a shift toward a type-2 polarization of the T cell compartment. However, this is purely speculative, as we could not determine from our cytokine data the mechanism of the asthma-like response that we observed on histology. The mechanism of CARDS toxin-mediated cytokine suppression remains unknown, but it might be more obvious in the baboon, as the kinetics of the T cell response to CARDS toxin or *M. pneumoniae* is slower than what is observed in the mouse.

The serologic response in this study was also variable. Although 75% of animals exposed to CARDS toxin showed increases in both toxin-specific IgM and IgG, animals exposed to *M. pneumoniae* showed more variability, with only 65% of animals developing significant levels of either IgM or IgG. However, all animals were initially negative for IgE directed to CARDS toxin, and 11/13 developed IgE to CARDS toxin by Day 14. Clinical studies have also shown a variable immunologic response to *M. pneumoniae*. One study in children with asthma with *M. pneumoniae* infection revealed higher levels of IgE and IL-9, and lower levels of IFN- γ (21). In contrast to these findings, another study showed no difference in IL-2, IFN- γ , or IL-4 levels in patients with *M. pneumoniae* infection (22). Nevertheless, in those patients with asthma symptoms and *M. pneumoniae* infection, IL-5 levels were found to be significantly higher compared with those with asymptomatic infection (21, 22). In line with these findings, an analysis of serum samples of subjects with asthma and concomitant *M. pneumoniae* infection revealed higher levels of IL-5 and IgE compared with patients with only *M. pneumoniae* infection (12). Therefore, even though the inflammatory patterns seen in patients exposed to *M. pneumoniae* are not uniform, there is strong evidence of a Th2 type response in a subset of patients.

The human pulmonary anatomy and physiology, compared with rodents and other small animals, differs significantly (23), and, therefore, nonhuman primate models are favored. Particularly in asthma, where there is an emphasis on the inflammatory mechanisms of disease, nonhuman primate models offer significant advantages to murine and other animal models. For example, leukotrienes and other important cytokines in asthma may

Figure 3. Proportion of peripheral blood intracellular IFN- γ (A) IL-17, (B) IL-4, and (C) in total T cells in baboons exposed to CARDS toxin (dotted lines) or *M. pneumoniae* at baseline, Day 7, and Day 14. (D) Shows the intercellular IL-4/IFN- γ ratio of peripheral blood cells at baseline, Day 7, and Day 14.

have minimal or no effect on airway inflammation in mice (24). In fact, compared with other phylogenetically similar nonhuman primates, the baboon exhibited more robust responses to leukotriene D₄ (24). Research baboons often live in corrals that provide environmental exposures to dust, unfiltered air, and other particulates similar to humans (25). In addition to these advantages, baboons are large enough to allow the use of medical equipment, such as bronchoscopes.

Similar to our baboon data, we reported that both adult and pediatric patients with asthma who test positive for CARDS toxin by PCR or antigen capture assays had variable serologic responses, with few patients exhibiting a robust response to CARDS toxin, and the majority displaying a minimal to low antibody response (11, 18). Similar patterns of serological responses are reflected in the baboon. Some animals exposed to recombinant CARDS toxin exhibited a robust antibody response over the 14-day course of the experiment,

whereas overall weaker responses were observed in animals infected with *M. pneumoniae*, similar to what we have observed in patients with asthma (11, 18). The exposure to *M. pneumoniae*, inciting a subclinical infection, may partially explain *M. pneumoniae*-associated exacerbation of asthma in humans and asthma-like pulmonary histopathologic changes in baboons. In our study, we demonstrate that, in baboons, exposure to *M. pneumoniae* did not produce a clinical infection, but resulted in an asthma-like inflammatory response. In addition, we were able to demonstrate the production of significant levels of CARDS toxin at Days 7 and 14 after instillation of *M. pneumoniae*. Although the bronchus intermedius appeared normal at the time of sampling the ELF, levels of toxin measured by antigen capture were similar to those reported to induce airway hyperresponsiveness in our murine models of CARDS toxin-induced asthma (20). The degree of histological severity correlated with levels of CARDS toxin by antigen capture on Day 7, but not on Day 14. This suggests that the initial degree of exposure may determine the degree of resulting inflammation. In CARDS toxin-exposed animals, the levels of toxin detected by antigen capture were mostly higher on Day 7 compared with Day 14. Despite this, the histopathologic severity was higher on Day 14. It is plausible that, by Day 14, toxin levels in the ELF were lower due to clearance mechanisms of airway secretions and local or systemic absorption. This suggests that CARDS toxin-induced inflammation may persist even after initial exposure. Because CARDS toxin binds to and is internalized by target cells, its persistence in the airways and continued ADP-ribosylating and vacuolating activities promote intracellular events that lead to increased histopathology and hyperinflammation (26). However, these observations are limited by the design of the experiment, given that only two bronchoscopic measurements of toxin levels were possible.

Evidence continues to emerge regarding the important association between *M. pneumoniae* infection and asthma. This study demonstrates that both

M. pneumoniae and CARDS toxin are capable of producing an allergic response in healthy, nonhuman primates with all of the histological features of asthma. Pulmonary instillation of *M. pneumoniae* resulted in the production of high levels of CARDS toxin by Day 7, despite the normal bronchoscopic appearance of the airway. The pathological features induced by *M. pneumoniae* and

CARDS toxin were very similar. Although there are likely multiple underlying pathologic mechanisms, the results of this study suggest that CARDS toxin plays a major role in the induction of an “asthma-like” histological response after exposure to *M. pneumoniae*. *M. pneumoniae* is the leading cause of community-acquired pneumonia among children worldwide. Furthermore, the alarming increase in

macrolide resistance and the absence of an effective vaccine further reinforce the need to better understand *M. pneumoniae* and CARDS toxin-mediated airway pathologies and their potential role in mediating or exacerbating chronic airway disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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